CELL-FREE SYNTHESIS OF POLYKETIDES

Description

Cross-Reference to Related Application

This application is related to provisional patent application serial no. 60/003,338, filed July 6, 1995, from which priority is claimed under 35 U.S.C. §119(e)(1) and which is incorporated herein by reference in its entirety.

Reference to Government Contract

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Technical Field

The present invention relates generally to polyketides and polyketide synthases. In particular, the invention pertains to novel methods of producing polyketides and libraries of polyketides using a cell-free system.

Background of the Invention

Polyketides are a large, structurally diverse family of natural products. Polyketides possess a broad range of biological activities including antibiotic and pharmacological properties. For example, polyketides are represented by such antibiotics as tetracyclines and erythromycin, anticancer agents including daunomycin,

immunosuppressants, for example FK506 and rapamycin, and
veterinary products such as monensin and avermectin.

Polyketides occur in most groups of organisms
and are especially abundant in a class of mycelial
5   bacteria, the actinomycetes, which produce various
polyketides. Polyketide synthases (PKSs) are
multifunctional enzymes related to fatty acid synthases
(FASs). PKSs catalyze the biosynthesis of polyketides
through repeated (decarboxylative) Claisen condensations
10   between acylthioesters, usually acetyl, propionyl,
malonyl or methylmalonyl. Following each condensation,
they introduce structural variability into the product by
catalyzing all, part, or none of a reductive cycle
comprising a ketoreduction, dehydration, and
15   enoylreduction on the β-keto group of the growing
polyketide chain. PKSs incorporate enormous structural
diversity into their products, in addition to varying the
condensation cycle, by controlling the overall chain
length, choice of primer and extender units and,
20   particularly in the case of aromatic polyketides,
regiospecific cyclizations of the nascent polyketide
chain. After the carbon chain has grown to a length
characteristic of each specific product, it is released
from the synthase by thiolysis or acyltransfer. Thus,
25   PKSs consist of families of enzymes which work together
to produce a given polyketide. It is the controlled
variation in chain length, choice of chain-building
units, and the reductive cycle, genetically programmed
into each PKS, that contributes to the variation seen
30   among naturally occurring polyketides.

Two general classes of PKSs exist. These
classifications are well known. See, for example,
Hopwood, D.A. and Khosla, C., Secondary Metabolites:
Their Function and Evolution (1992) Wiley Chichester
35   (Ciba Foundation Symposium 171) pp. 88-112.

One class, known as Type I or modular PKSs, is
represented by the PKSs which catalyze the biosynthesis
of complex polyketides such as erythromycin and
avermectin. These "modular" PKSs include assemblies of
5   several large multifunctional proteins carrying, between
them, a set of separate active sites for each step of
carbon chain assembly and modification (Cortes, J. et al.
Nature (1990) 348:176; Donadio, S. et al. Science (1991)
252:675; MacNeil, D.J. et al. Gene (1992) 115:119). The
10   active sites required for one cycle of condensation and
reduction are clustered as "modules" (Donadio et al.
Science (1991), supra; Donadio, S. et al. Gene (1992)
111:51). For example, 6-deoxyerythronolide B synthase
(DEBS) consists of the three multifunctional proteins,
15   DEBS 1, DEBS 2, and DEBS 3 (Caffrey, P. et al. FEBS
Letters (192) 304:225), each of which possesses two
modules. (See Figure 1.)

As described below, a module contains at least
the minimal activities required for the condensation of
20   an extender unit onto a growing polyketide chain; the
minimal activities required are a ketosynthase (KS), an
acyl transferase (AT) and an acyl carrier protein (ACP).
Additional activities for further modification reactions
such as a reductive cycle or cyclization may also be
25   included in a module. Structural diversity occurs in
this class of PKSs from variations in the number and type
of active sites in the PKSs. This class of PKSs displays
a one-to-one correlation between the number and
clustering of active sites in the primary sequence of the
30   PKS and the structure of the polyketide backbone.

The second class of PKSs, the aromatic or Type
II PKSs, has a single set of iteratively used active
sites (Bibb, M.J. et al. EMBO J. (1989) 8:2727; Sherman,
D.H. et al. EMBO J. (1989) 8:2717; Fernandez-Moreno, M.A.
35   et al. J. Biol. Chem. (1992) 267:19278). Streptomyces is

an actinomycete which is an abundant producer of aromatic polyketides. In each *Streptomyces* aromatic PKS so far studied, carbon chain assembly requires the products of three open reading frames (ORFs). (See Figure 2.) ORF1

5   encodes a ketosynthase (KS) and an acyltransferase (AT) active site (KS/AT); ORF2 encodes a chain length determining factor (CLF), a protein similar to the ORF1 product but lacking the KS and AT motifs; and ORF3 encodes a discrete acyl carrier protein (ACP). Some gene

10   clusters also code for a ketoreductase (KR) and a cyclase, involved in cyclization of the nascent polyketide backbone. However, it has been found that only the KS/AT, CLF, and ACP, need be present in order to produce an identifiable polyketide.

15   Fungal PKSs, such as the 6-methylsalicylic acid PKS, consist of a single multidomain polypeptide which includes all the active sites required for the biosynthesis of 6-methylsalicylic acid (Beck, J. *et al.* *Eur. J. Biochem.* (1990) 192:487-498; Davis, R. *et al.*

20   *Abstr. of the Genetics of Industrial Microorganism Meeting, Montreal, abstr.* P288 (1994)). Fungal PKSs incorporate features of both modular and aromatic PKSs.

Streptomyces coelicolor produces the blue-pigmented polyketide, actinorhodin. The

25   actinorhodin gene cluster (*act*), has been cloned (Malpartida, F. and Hopwood, D.A. *Nature* (1984) 309:462; Malpartida, F. and Hopwood, D.A. *Mol. Gen. Genet.* (1986) 205:66) and completely sequenced (Fernandez-Moreno *et al.* *J. Biol. Chem.* (1992), *supra*; Hallam, S.E. *et al.* *Gene*

30   (1988) 74:305; Fernandez-Moreno, M.A. *et al.* *Cell* (1991) 66:769; Caballero, J. *et al.* *Mol. Gen. Genet.* (1991) 230:401). The cluster encodes the PKS enzymes described above, a cyclase and a series of tailoring enzymes involved in subsequent modification reactions leading to

35   actinorhodin, as well as proteins involved in export of

the antibiotic and at least one protein that specifically activates transcription of the gene cluster. Other genes required for global regulation of antibiotic biosynthesis, as well as for the supply of starter

5   (acetyl-CoA) and extender (malonyl-CoA) units for polyketide biosynthesis, are located elsewhere in the genome.

The *act* gene cluster from *S. coelicolor* has been used to produce actinorhodin in *S. parvulus*.

10 Malpartida, F. and Hopwood, D.A. *Nature* (1984) 309:462.

Bartel *et al. J. Bacteriol.* (1990) 172:4816-4826, recombinantly produced aloesaponarin II using *S. galilaeus* transformed with an *S. coelicolor act* gene cluster consisting of four genetic loci, *actI*,

15 *actIII*, *actIV* and *actVII*. Hybrid PKSs, including the basic *act* gene set but with ACP genes derived from granaticin, oxytetracycline, tetracenomycin and frenolicin PKSs, have also been designed which are able to express functional synthases. Khosla, C. *et al. J.*

20 *Bacteriol.* (1993) 175:2197-2204. Hopwood, D.A. *et al. Nature* (1985) 314:642-644, describes the production of hybrid aromatic polyketides, using recombinant techniques. Sherman, D.H. *et al. J. Bacteriol.* (1992) 174:6184-6190, reports the transformation of various *S.*

25 *coelicolor* mutants, lacking different components of the *act* PKS gene cluster, with the corresponding granaticin (*gra*) genes from *S. violaceoruber*, in trans.

Although the above described model for complex polyketide biosynthesis by modular (Type I) PKSs has been

30 substantiated by radioisotope and stable isotope labeling experiments, heterologous expression, directed mutagenesis, and *in vitro* studies on partially active proteins, cell-free enzymatic synthesis of complex polyketides has proved unsuccessful despite more than 30

35 years of intense efforts (Caffrey *et al. FEBS Letters*

(1992), *supra*; Aparicio, J.F. *et al.* *J. Biol. Chem.*
(1994) 269:8524; Bevitt, D.J. *et al.* *Eur. J. Biochem.*
(1992) 204:39; Caffrey, P. *et al.* *Eur. J. Biochem.* (1991)
195:823); Leadlay, P.F. *et al.* *Biochem. Soc. Trans.*

5 (1993) 21:218; Marsden, A.F.A. *et al.* *Science* (1994)
263:378; Wawszkiewicz, E.J. *et al.* *Biochemische Z.* (1964)
340:213; Corcoran, J.W. *et al.* in *Proc. 5th Int. Congr.
Chemother.* (Vienna, 1967), Abstracts of Communications,
p. 35; Corcoran, J.W. *et al.* in *Antibiotics IV.*

10 *Biosynthesis* (1982) Corcoran, J.W., Ed. (Springer-Verlag,
New York) p. 146; Roberts, G. *FEBS Lett.* (1983) 159:13;
Roberts, G. *et al.* *Biochemical Soc. Trans.* (1984) 12:642;
Hunaiti, A.A. *et al.* *Antimicrob. Agents. Chemother.*
(1984) 25:173). This is due, in part, to the difficulty

15 of isolating fully active forms of these large, poorly
expressed multifunctional proteins from naturally
occurring producer organisms and, in part, to the
relative lability of intermediates formed during the
course of polyketide biosynthesis. For example, the

20 three DEBS proteins have been purified individually from
the natural producer organism, *Saccharopolyspora
erythraea* (Caffrey *et al.* *FEBS Letters* (1992), *supra*;
Aparicio *et al.* *J. Biol. Chem.* (1994), *supra*; Bevitt *et
al.* *Eur. J. Biochem.* (1992), *supra*; Caffrey *et al.* *Eur.
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25 *J. Biochem.* (1991), *supra*; Leadlay *et al.* *Biochem. Soc.
Trans.* (1993); Marsden *et al.* *Science* (1994), *supra*).
Studies on the purified enzymes facilitated clarification
of their stereospecificity, showing that
2*S*-methylmalonyl-CoA is the extender substrate for all 6

30 acyltransferase sites (Marsden *et al.* *Science* (1994),
supra), thereby implying that the differing
configurations of the methyl-branched centers result from
selective epimerization of specific enzyme-bound
intermediates. However, the lack of a full turnover

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assay prevented these investigators from probing the mechanisms of the enzyme complex in greater detail.

In an attempt to overcome some of these limitations, modular PKS subunits have been expressed in heterologous hosts such as *E. coli* (Aparicio *et al. J. Biol. Chem.* (1994), *supra*; Bevitt *et al. Eur. J. Biochem.* (1992), *supra*; Caffrey *et al. Eur. J. Biochem.* (1991), *supra*; Leadlay *et al. Biochem. Soc. Trans.* (1993);) and *S. coelicolor* (Kao, C.M. *et al. Science* (1994) 265:509; International Publication No. WO 95/08548 (published March 30, 1995)). Whereas the proteins expressed in *E. coli* are not fully active, heterologous expression in *S. coelicolor* resulted in production of active protein as demonstrated by the production of 6-deoxyerythronolide ("6-DEB") *in vivo*. Cell-free enzymatic synthesis of polyketides from simpler PKSs such as the 6-methylsalicylate synthase (Dimroth, P. *et al. Eur. J. Biochem.* (1970) 13:98; Beck, J. *et al. Eur. J. Biochem.* (1990) 192:487); Spencer J.B. *et al. Biochem. J.* (1992) 288:839), chalcone synthase (Lanz, T. *et al. J. Biol. Chem.* (1991) 266:9971 (1991)), and the tetracenomycin synthase (Shen, B. *et al. Science* (1993) 262:1535) has been reported.

However, no one to date has described the cell-free enzymatic synthesis of polyketides from modular PKSs, or has used a cell-free system to produce libraries containing a multiplicity of different polyketides.

Summary of the Invention

The present invention provides methods to produce both novel and known polyketides. In one embodiment, a cell-free system comprising a modular PKS effects synthesis of a polyketide when incubated with an appropriate substrate set.

In another embodiment, the invention is directed to a method of synthesizing a library containing a multiplicity of different polyketides by use of cell-free systems and to a matrix of cell-free subsystems for the production of these libraries.

Thus, in one aspect, the invention is directed to a method comprising providing one or more proteins comprising at least two modules of a modular polyketide synthase in a cell-free system; adding to said system at least one starter unit and at least one extender unit; incubating said cell-free system containing said starter unit and extender unit under conditions wherein said polyketide is synthesized; and optionally recovering the polyketide from the cell-free system.

In another aspect, the invention is directed to a matrix for the production of a polyketide library which comprises a series of cell-free subsystems each containing one or more polyketide synthase proteins comprising enzymatic activities that effect the coupling of at least one extender unit to a starter unit, including a growing polyketide chain; each said subsystem containing at least one starter unit and at least one extender unit; and wherein at least one enzymatic activity or at least one extender unit or at least one starter unit or is different as between each subsystem.

The invention in another aspect is directed to methods to prepare libraries of polyketides using these matrices.

In yet another aspect, the invention is directed to method to produce a desired polyketide which method comprises: providing a system comprising a functional modular polyketide synthase (PKS), or a functional portion thereof, wherein said PKS cannot be loaded with a natural first-module starter unit, or wherein, once loaded, cannot catalyze the condensation of

an extender unit to the first-module starter unit to produce a polyketide intermediate; adding to said system a starter unit that is a substrate for the PKS; incubating the system containing said PKS and said

5   starter unit substrate under conditions wherein said polyketide is synthesized; and optionally recovering the polyketide.

In still another aspect, the invention is directed to a functional modular polyketide synthase

10   system, or a functional portion thereof, which cannot be loaded with a natural first-module starter unit, or which, once loaded, cannot catalyze the condensation of an extender unit to the first-module starter unit to produce a polyketide intermediate.

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Brief Description of the Figures

Figure 1 is a diagram of the organization of the modular PKS cluster which catalyzes the production of 6-DEB (1).

20   Figure 2 diagrams a typical aromatic PKS gene cluster.

Figure 3A depicts a Coomassie Blue-stained 5% acrylamide gel of protein fractions containing DEBS 1+2+thioesterase (DEBS 1+2+TE) from pCK12 or the complex

25   of DEBS 1, 2, and 3 from pCK7. Lanes Ia, Ib and Ic show DEBS 1+2+TE after purification Step 1 (a), after Step 2 (b) and after Step 3 (c). Lanes IIa, IIb, IIc, IId and IIe show DEBS 1, 2, and 3 after Step 1 (a), after Step 2 (b), after Step 3 (c), (d) and (e) in order of increasing

30   elution volume.

Figure 3B depicts an autoradiogram showing the covalent modification of DEBS 1, 2, and 3 (Lanes I a-d) or DEBS 1+2+TE (Lanes II a-c) by ^{14}C-labeled starter units [1-^{14}C]propionyl-CoA (20 μM) (Lanes Ia and IIa),

35   [1-^{14}C]butyryl-CoA (160 μM) (Lanes Ib and IIb),

[1-^{14}C]acetyl-CoA (40 μM) (Lanes Ic and IIc) and
[1-^{14}C]propionyl-CoA (20 μM) after a 30 min-preincubation
with iodoacetamide (1 Mm) (Lane Id).

      Figure 4 depicts an autoradiogram showing *in*
5   *vitro* synthesis of ^{14}C-labeled 6-DEB (**1**) and the
triketide lactone (**2**).

      Figure 5 is a pictorial representation of the
conversion of [1-^{13}C] propionyl-CoA to triketide lactone
(**2a**) by DEBS 1+2+TE (a) and the conversion of
10   [2,3-^{13}C$_2$]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-NAC
thioester (**3**) triketide lactone (**2b**) by DEBS 1+2+TE (b).

      Figure 6A depicts a thin layer chromatography-
autoradiogram showing the incorporation of alternative
substrates into polyketide products. Lane Ib contains
15   the putative C$_{10}$-lactone homolog (**5**) (*see* Figure 5B), R$_f$
= 0.36 (marked by arrow 1). Lane IIb contains (**4**) (*see*
Figure 5B), R$_f$ = 0.23, (marked by arrow 2, identical to
the R$_f$ of an authentic sample of compound (**4**).

      Figure 6B is a pictorial representation of the
20   conversion of acetyl-CoA to compound (**4**) by DEBS 1+2+TE
(a) and the proposed conversion of butyryl-CoA to
compound (**5**) by DEBS 1+2+TE (b).

Detailed Description of the Invention

25   The invention provides cell-free systems for
the synthesis of novel and known polyketides and of
polyketide libraries. In the case of modular polyketide
synthases, cell-free production of polyketides using
these enzymes has not heretofore been accomplished.
30   Although, as described above, cell-free synthesis of
polyketides by some aromatic synthases has been achieved,
these systems have not been used in constructing
libraries of polyketides, said libraries being useful as
sources of compounds to be screened for pharmacological
35   or other activities.

The use of cell-free systems for the
construction of such libraries has several advantages.
First, permeability problems are eliminated, so that
substrates can be used which might otherwise be

5   ineffective due to failure to permeate the cell. Second,
variability in product due to differential permeability
is eliminated. Third, alternative metabolic events are
minimized or eliminated so that the reaction proceeds
cleanly to convert substrates to polyketide products.

10 Fourth, there are greater possibilities for regulating
the conditions under which the polyketide synthase genes
are expressed and the polyketides are produced. For
example, cofactors which are ordinarily useful in the
synthesis of a given polyketide, such as NADPH, can be

15 supplied or withheld. Finally, it is possible to use
"unnatural" substrates for a given synthase since
cellular mechanisms for providing the substrate to the
synthase are eliminated. As a result of using cell-free
systems to create libraries, a greater variety of

20 polyketides may be synthesized than would have been
possible had production been limited to intracellular
synthesis.

        Given a particular cell-free system containing
polyketide synthase proteins, the nature of the

25 polyketide ultimately produced will depend on the
substrates provided and on the conditions with respect to
cofactors, etc. In order to explore the possibilities
for a given cell-free system with a given complement of
PKS proteins, it will be advantageous to subdivide the

30 cell-free system into "subsystems" with variation in
these factors. In order to be workable, the cell-free
system or subsystem must contain polyketide synthase
proteins with enzymatic activities sufficient to effect
the condensation of an "extender unit" onto a "starter

35 unit," where a "starter unit" may include a growing

polyketide chain. Because the cell-free system offers greater promiscuity of starter and extender units, a number of subsystems containing a variety of starter and extender units, as well as differing conditions may

5   result in a corresponding variety of polyketides.

As used in the present application, a "starter unit" refers to a substance to which additional Claisen condensations may be effected. The starter unit may be one which is natively regarded as a starter, or may be

10   what would in the native state be an intermediate growing polyketide chain. A "first-module starter unit" is an acyl thioester that can be loaded onto the appropriate active site of the first module of a PKS. A "natural starter unit" is an acyl thioester which upon extension

15   by the PKS produces the natural polyketide product. An "unnatural starter unit" is an acyl thioester which upon extension by the PKS produces a polyketide product other than that normally produced by the PKS during metabolism.

A relaxed specificity of modular PKS for

20   starter units under *in vitro* conditions has been reported by Pieper *et al.* *Nature* (1995) 378:263-266. Known starter units include, for example acetyl-CoA, propionyl-CoA, butyryl-CoA, isobutyryl-CoA, cyclohexanoyl-CoA, aminohydroxy benzoyl-CoA, and intermediate polyketide

25   chains. In addition, an extender unit may be used as a source of starter units (*see* Pieper *et al.* *Biochem.* (1996) 35:2054-2060). Thus, in a system capable of producing a polyketide, the starter unit and the extender unit used therein may be the same or different.

30   The starter unit is then extended by virtue of the activity of the synthase contained in the cell-free system or subsystem. Extender units are added to the carboxy terminus of a growing polyketide and the nature of the extender unit is determined by the acyl

35   transferase (AT) activity. Suitable extender units

include malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-
CoA. Sequence comparisons have identified the
characteristics of malonyl-CoA-specific AT and
methylmalonyl-CoA-specific AT (Haydock *et al.* *FEBS Lett.*

5   (1995) 374:246-248. When methylmalonyl-CoA or
ethylmalonyl-CoA is used as the extender unit, a chiral
center is generated in the condensation.

The reductive cycle which occurs in either
aromatic or modular PKS systems depends both on the

10   presence of suitable ketoreductase (KR) activity as well
as the reaction conditions in an *in vitro* system. The
absence of reductive activity yields a ketone; reduction
generates an alcohol containing a chiral center. If a
dehydratase (DH) activity is also present, an alkene

15   results which eliminates the chiral center.
Additionally, an enoyl reductase activity may be present
which reduces the β-keto group to a methylene group.
Thus, for a single condensation of an extender unit,
there are five theoretically possible reductive cycle

20   outcomes. This variation is effected both by the
cofactor conditions and by the nature of the proteins in
the cell-free system to be employed.

Various other catalytic activities resulting in
cyclization, aromatization, chain-length limitation, and

25   the like are determined mainly by the nature of the
synthase proteins.

Thus, the availability of cell-free systems for
the production of ketides provides a unique opportunity
to generate libraries of polyketides by varying the

30   nature of the synthase, the nature of the extender unit,
the nature of the starter unit and the nature of the
conditions. A simple matrix can be envisioned whereby
cell-free systems containing varying synthase catalytic
activities, but at a minimum the capability to extend a

35   starter unit, including a growing polyketide chain

through an additional Claisen condensation, can be
employed. Each of these cell-free systems can be
subdivided into subsystems in which the remaining
variables are manipulated to effect the eventual outcome
5   of synthesis. Thus, a series of subsystems containing
identical polyketide synthase activities can be supplied
different starter units, different extender units, and
incubated under different conditions so as to result in a
multiplicity of polyketides. Similar variation can be
10   employed with respect to subsystems of cell-free systems
containing different PKS activities, thus resulting in a
matrix wherein one dimension may be envisioned as varying
the nature of the cell-free system itself and the other
dimension comprises variation in the substrates and
15   conditions.

A.   Definitions
In describing the present invention, the
following terms will be employed, and are intended to be
20   defined as indicated below.
By a "cell-free system" is intended a cell
lysate, cell extract or other preparation in which
substantially all of the cells in the preparation have
been disrupted or otherwise processed so that all or
25   selected cellular components, e.g., organelles, proteins,
nucleic acids, the cell membrane itself (or fragments or
components thereof), or the like, are released from the
cell or resuspended into an appropriate medium and/or
purified from the cellular milieu. Cell-free systems
30   include, of course, reaction mixtures prepared from
purified or isolated proteins and suitable reagents and
buffers.
By a "cell-free subsystem" is meant either a
portion of a cell-free system -- i.e., the cell-free
35   system that results when a given composition is

effectively subdivided into two or more separate
compartments for independent catalysis, or is a reaction
mixture which contains the same complement of polyketide
synthase enzymatic activity. Thus, a "subsystem" of a

5   given "cell-free system" may differ in composition by
virtue of differing substrates or conditions, but
contains the same catalytic polyketide synthase
activities.

By "purified" or "isolated" is meant, when

10   referring to a polypeptide or nucleotide sequence, that
the indicated molecule is separate and discrete from the
whole organism from which the molecule is normally
associated in nature. Thus, a protein contained in a
cell free extract would constitute a "purified" or

15   "isolated" protein, as would a protein further purified
from a cell-free extract. In addition, a "purified" or
"isolated" protein refers to a protein which has been
synthetically or recombinantly produced and, optionally,
purified from the host cell. An "isolated" nucleotide

20   sequence is a nucleotide sequence separate and discrete
from the whole organism with which the sequence is found
in nature; or a sequence devoid, in whole or part, of
sequences normally associated with it in nature; or a
sequence, as it exists in nature, but having heterologous

25   sequences (as defined below) in association therewith.

A single "module" of a modular PKS gene cluster
or a modular polyketide synthase refers to sufficient
portions of the gene cluster to encode, or sufficient
portions of the polyketide synthase to include, at least

30   the activities required to effect the condensation of a
single extender unit onto a starter unit or a growing
polyketide chain. Thus, the minimal activities required
include a ketosynthase (KS) an acyltransferase (AT) and
an acyl carrier protein (ACP). All three of these

35   activities are required for the condensation of a single

extender unit onto the growing polyketide chain. At
least one module for the effective synthesis of a
polyketide must contain an additional AT and ACP in order
to effect the initial condensation. In addition, and
5   optionally, the module may include a ketoreductase
activity (KR), a cyclase, a dehydratase (DH) an enoyl
reductase (ER) and/or a thioesterase (TE).

      In native forms of aromatic polyketide
synthases, portions of the required activities may occur
10   on different proteins. In the case of aromatic
polyketide synthases also, a ketosynthase (KS), an acyl
transferase (AT) and an acyl carrier protein (ACP) must
be present to effect the condensation of a single
extender unit onto a starter unit or a growing
15   polyketide. Various activities associated with
reduction, cyclization, aromatization and further
derivatization may also be present. There must also be
at least one chain-length limiting factor (CLF).

      The phrases "PKS gene cluster" and "PKS gene
20   set" are used interchangeably to mean any set of PKS
genes capable of producing a functional PKS when under
the direction of one or more compatible control elements,
as defined below, in a host cell. A functional PKS is
one which catalyzes the condensation of at least one
25   extender unit onto a growing polyketide -- i.e., has at
least one functional module, or extension function either
in vivo or *in vitro*. A "PKS gene cluster" thus need not
include all of the genes found in the corresponding
cluster in nature.

30   Furthermore, the cluster can include PKS genes
derived from a single species, or may be hybrid in nature
with, e.g., a coding sequence derived from a cluster for
the synthesis of a particular polyketide replaced with a
corresponding coding sequence from a cluster for the
35   synthesis of another polyketide. Hybrid clusters can

include genes derived from either or both modular and
aromatic PKSs. The genes included in the gene cluster
need not be the native genes, but can be mutants or
analogs thereof. Mutants or analogs may be prepared by
5   the deletion, insertion or substitution of one or more
nucleotides of the coding sequence. Techniques for
modifying nucleotide sequences, such as site-directed
mutagenesis, are described in, e.g., Sambrook et al.,
supra; DNA Cloning, Vols. I and II, supra; Nucleic Acid
10   Hybridization, supra.

         A "PKS gene cluster" may also contain genes
coding for modifications to the core polyketide produced
by the PKS, including, for example, genes encoding
post-polyketide synthesis enzymes derived from natural
15   products pathways such as O-methyltransferases and
glycosyltransferases. A "PKS gene cluster" may further
include genes encoding hydroxylases, methylases or other
alkylases, oxidases, reductases, glycotransferases,
lyases, ester or amide synthases, and various hydrolases
20   such as esterases and amidases.

         As explained further below, the genes included
in the PKS gene cluster need not be on the same plasmid
or, if present on the same plasmid, can be controlled by
the same or different control sequences.

25   A "host cell" is a cell and the progeny and
cultures thereof derived from a procaryotic microorganism
or a eucaryotic cell line cultured as a unicellular
entity, which can be, or has been, used as a recipient
for recombinant vectors bearing the PKS gene clusters of
30   the invention. It is understood that the progeny of a
single parental cell may not necessarily be completely
identical in morphology or in genomic or total DNA
complement to the original parent, due to accidental or
deliberate mutation. Progeny of the parental cell which
35   are sufficiently similar to the parent to be

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characterized by the relevant property, such as the
presence of a nucleotide sequence encoding a desired PKS,
are included in the definition, and are covered by the
above terms.

5   The term "heterologous" as it relates to
nucleic acid sequences such as coding sequences and
control sequences, denotes sequences that are not
normally associated with a region of a recombinant
construct, and/or are not normally associated with a
10   particular cell. Thus, a "heterologous" region of a
nucleic acid construct is an identifiable segment of
nucleic acid within or attached to another nucleic acid
molecule that is not found in association with the other
molecule in nature. For example, a heterologous region
15   of a construct could include a coding sequence flanked by
sequences not found in association with the coding
sequence in nature. Another example of a heterologous
coding sequence is a construct where the coding sequence
itself is not found in nature (e.g., synthetic sequences
20   having codons different from the native gene).
Similarly, a host cell transformed with a construct which
is not normally present in the host cell would be
considered heterologous for purposes of this invention.
Allelic variation or naturally occurring mutational
25   events do not give rise to heterologous DNA, as used
herein.

A "coding sequence" or a sequence which
"encodes" a protein or peptide is a nucleic acid sequence
which is transcribed into mRNA (in the case of DNA) or
30   translated into a polypeptide (in the case of mRNA) *in
vitro* or *in vivo* when placed under the control of
appropriate regulatory sequences.

"Control sequences" refers collectively to
promoter sequences, ribosome binding sites,
35   polyadenylation signals, transcription termination

sequences, upstream regulatory domains, enhancers, and
the like, which collectively provide for the
transcription and/or translation of a coding sequence in
a host cell. Not all of these control sequences need

5   always be present in a recombinant vector so long as the
desired gene is capable of being transcribed and
translated.

"Operably linked" refers to an arrangement of
elements wherein the components so described are

10   configured so as to perform their usual function. Thus,
control sequences operably linked to a coding sequence
are capable of effecting the expression of the coding
sequence. The control sequences need not be contiguous
with the coding sequence, so long as they function to

15   direct the expression thereof. Thus, for example,
intervening untranslated yet transcribed sequences can be
present between a promoter sequence and the coding
sequence and the promoter sequence can still be
considered "operably linked" to the coding sequence.

20   A "library" or "combinatorial library" of
polyketides is intended to mean a collection of a
multiplicity of different polyketides. The differences
in the members of the library may result from their being
produced by different PKS cell-free systems that contain

25   any combination of native, homolog or mutant genes from
aromatic, modular or fungal PKSs. The differences in the
members of the library may also result from the use of
different starter units, extender units and conditions.
The PKSs in the cell-free systems used to generate the

30   library may be derived from a single system, such as *act*,
fren, *gra*, *tcm*, *whiE*, *gris*, *ery*, or the like, and may
optionally include genes encoding tailoring enzymes which
are capable of catalyzing the further modification of a
polyketide. Alternatively, the combination of synthase

35   activities can be rationally or stochastically derived

from an assortment of synthases, e.g., a synthase system
can be constructed to contain the KS/AT component from an
act PKS, the CLF component from a *gra* PKS and a ACP
component from a *fren* PKS. The synthase can optionally
5   include other enzymatic activities as well.

The variety of polyketides in the library may
thus result from varying the nature of the synthase or
varying the nature of the substrates used to construct
the polyketides or both. Preferably, the library is
10   produced as a result of culturing a matrix which varies
the nature of the synthase systems in one dimension and
the nature of the substrates and/or incubation conditions
in the other. The library of polyketides thus produced
can be tested or screened for biological, pharmacological
15   or other activity.

"Optional" or "optionally" means that the
subsequently described event or circumstance may or may
not occur, and that the description includes instances
where said event or circumstance occurs and instances
20   where it does not. For example, the phrase "optionally
further purified" means that further purification may or
may not be performed and that the description includes
both the performance and the lack of performance of such
further purification.

25

B.   General Methods

The polyketides produced by the invention
methods can be screened for use as therapeutic agents to
treat a number of disorders, depending on the type of
30   polyketide in question. For example, several of the
polyketides produced by the present method will find use
as immunosuppressants, as anti-tumor agents, as well as
for the treatment of viral, bacterial and parasitic
infections.

35

By use of the cell-free systems of the
invention, a wide variety of polyketides can be
synthesized as candidates. As explained above, the use
of cell-free technology permits greater flexibility in
5   choice of substrate and less interference in the
synthesis of the desired polyketide from competing
metabolic reactions. The ability to produce polyketides
in a cell-free system also provides a powerful tool for
characterizing PKSs and the mechanism of their actions.
10   The practice of the present invention will
employ, unless otherwise indicated, conventional methods
of chemistry, microbiology, molecular biology and
recombinant DNA techniques within the skill of the art.
Such techniques are explained fully in the literature.
15   See, e.g., Sambrook, et al. *Molecular Cloning: A
Laboratory Manual* (Current Edition); *DNA Cloning: A
Practical Approach*, vol. I & II (D. Glover, ed.);
Oligonucleotide Synthesis (N. Gait, ed., Current
Edition); *Nucleic Acid Hybridization* (B. Hames & S.
20   Higgins, eds., Current Edition); *Transcription and
Translation* (B. Hames & S. Higgins, eds., Current
Edition); H.O. House, *Modern Synthetic Reactions*, Second
Edition (Menlo Park, CA: The Benjamin/Cummings Publishing
Company, 1972); and J. March, *Advanced Organic Chemistry:
25   Reactions, Mechanisms and Structure*, 4th Ed. (New York:
Wiley-Interscience, 1992).

All publications, patents and patent
applications cited herein, whether *supra* or *infra*, are
hereby incorporated by reference in their entirety.
30   As used in this specification and the appended
claims, the singular forms "a," "an" and "the" include
plural references unless the content clearly dictates
otherwise. Thus, reference to "a polyketide synthase"
includes mixtures of polyketide synthases, reference to

35

"a PKS enzyme" includes mixtures of such enzymes, and the like.

1.   Recombinant Production of PKS

5   The invention, for the production and isolation of a significant quantity of functional modular PKS enzymes, in particular, makes use of host cells transformed with recombinant vectors for the production of these enzymes. Aromatic and hybrid PKS may be

10   produced in this way as well. The host cells may be genetically engineered cells which have their naturally occurring PKS genes substantially deleted.

Host cells for the production of the functional PKS enzymes effective in cell-free systems can be derived

15   from any organism with the capability of harboring a recombinant PKS gene cluster, and can be derived from either procaryotic or eucaryotic organisms. However, preferred host cells are those constructed from the actinomycetes, a class of mycelial bacteria which are

20   abundant producers of a number of polyketides. A particularly preferred genus for use in production of the PKSs is *Streptomyces*. Thus, for example, *S. ambofaciens, S. avermitilis, S. azureus, S. cinnamonensis, S. coelicolor, S. curacoi, S. erythraeus, S. fradiae, S.*

25   *galilaeus, S. glaucescens, S. hygroscopicus, S. lividans, S. parvulus, S. peucetius, S. rimosus, S. roseofulvus, S. thermotolerans, S. violaceoruber,* among others, will provide convenient host cells, with *S. coelicolor* being preferred. (*See, e.g.,* Hopwood, D.A.

30   and Sherman, D.H. *Ann. Rev. Genet.* (1990) <u>24</u>:37-66; O'Hagan, D. *The Polyketide Metabolites* (Ellis Horwood Limited, 1991), for a description of various polyketide-producing organisms and their natural products.)

35

The above-described cells can be genetically engineered by deleting the naturally occurring PKS genes therefrom, using standard techniques, such as by homologous recombination. (*See, e.g.*, Khosla, C. *et al*.

5   *Molec. Microbiol.* (1992) 6:3237). For example, native strains of *S. coelicolor* produce a PKS which catalyzes the biosynthesis of the aromatic polyketide actinorhodin. The strain, *S. coelicolor* CH999 (as described in WO 95/08548, *supra*), was constructed by deleting, via

10   homologous recombination, the entire natural *act* cluster from the chromosome of *S. coelicolor* CH1 (Khosla *et al*. *Molec. Microbiol.* (1992), *supra*), a strain lacking endogenous plasmids and carrying a stable mutation that blocks biosynthesis of another pigmented *S. coelicolor*

15   antibiotic, undecylprodigiosin.

The host cells described above can be transformed with one or more vectors, collectively encoding at least a set of functional PKS activities sufficient to effect condensation of an extender unit, or

20   a cocktail comprising a random assortment of PKS associated sequences with this activity. The vector(s) can include native or hybrid combinations of PKS subunits or cocktail components, or mutants thereof.

In order to produce the PKS for practice of the

25   cell-free synthesis, recombinant vector(s) can be constructed that include genes from a single PKS aromatic or modular gene cluster, or may comprise hybrid PKS gene clusters with, e.g., a gene or part of a gene from one cluster replaced by the corresponding portion from

30   another gene cluster. For example, it has been found that ACPs are readily interchangeable among different aromatic synthases without an effect on product structure. Furthermore, a given KR can recognize and reduce polyketide chains of different chain lengths.

35   Accordingly, these coding sequences are freely

interchangeable in the constructs described herein.
Thus, the PKS gene clusters used to produce the PKS
enzymes can be derived from any combination of PKS gene
sequences which ultimately function to produce a PKS that

5   condenses at least one extender unit into a growing
polyketide.

Examples of hybrid clusters include clusters
with coding sequences derived from two or more of the *act*
gene cluster, the *whiE* gene cluster, frenolicin (*fren*),

10 granaticin (*gra*), tetracenomycin (*tcm*), 6-methylsalicylic
acid (*6-msas*), oxytetracycline (*otc*), tetracycline (*tet*),
erythromycin (*ery*), griseusin (*gris*), nanaomycin,
medermycin, daunorubicin, tylosin, carbomycin,
spiramycin, avermectin, monensin, nonactin, curamycin,

15 rifamycin and candicidin synthase gene clusters, among
others. A number of hybrid gene clusters have been
constructed having components derived from the *act*, *fren*,
tcm, *gris* and *gra* gene clusters (see, WO 95/08548).
Several of the hybrid clusters were able to functionally

20 express both novel and known polyketides in *S. coelicolor*
CH999. However, other hybrid gene clusters, as described
above, can easily be produced and screened using the
disclosure herein, for the production of identifiable
polyketides.

25 The recombinant vectors, harboring the gene
clusters or random assortment of PKS genes, modules,
active sites or portions thereof described above, can be
conveniently generated using techniques known in the art.
For example, the PKS subunits of interest can be obtained

30 from an organism that expresses the same, using
recombinant methods, such as by screening cDNA or genomic
libraries, derived from cells expressing the gene, or by
deriving the gene from a vector known to include the
same. The gene can then be isolated and combined with

35 other desired PKS subunits, using standard techniques.

If the gene in question is already present in a suitable
expression vector, it can be combined *in situ*, with,
e.g., other PKS subunits, as desired. The gene of
interest can also be produced synthetically, rather than
5   cloned. The nucleotide sequence can be designed with the
appropriate codons for the particular amino acid sequence
desired. In general, one will select preferred codons
for the intended host in which the sequence will be
expressed. The complete sequence can be assembled from
10   overlapping oligonucleotides prepared by standard methods
and assembled into a complete coding sequence. *See,*
e.g., Edge (1981) *Nature* 292:756; Nambair *et al.* (1984)
Science 223:1299; Jay *et al.* (1984) *J. Biol. Chem.*
259:6311.
15   Mutations can be made to the native PKS subunit
sequences and such mutants used in place of the native
sequence, so long as the mutants are able to function
with other PKS subunits to collectively catalyze the
synthesis of an identifiable polyketide. Such mutations
20   can be made to the native sequences using conventional
techniques such as by preparing synthetic
oligonucleotides including the mutations and inserting
the mutated sequence into the gene encoding a PKS subunit
using restriction endonuclease digestion. (See, *e.g.,*
25   Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* 82:448;
Geisselsoder *et al.* (1987) *BioTechniques* 5:786.)
Alternatively, the mutations can be effected using a
mismatched primer (generally 10-20 nucleotides in length)
which hybridizes to the native nucleotide sequence
30   (generally cDNA corresponding to the RNA sequence), at a
temperature below the melting temperature of the
mismatched duplex. The primer can be made specific by
keeping primer length and base composition within
relatively narrow limits and by keeping the mutant base
35   centrally located. Zoller and Smith, *Methods Enzymol.*

(1983) 100:468. Primer extension is effected using DNA
polymerase, the product cloned and clones containing the
mutated DNA, derived by segregation of the primer
extended strand, selected. Selection can be accomplished

5   using the mutant primer as a hybridization probe. The
technique is also applicable for generating multiple
point mutations. *See, e.g.*, Dalbie-McFarland *et al.*
Proc. Natl. Acad. Sci USA (1982) 79:6409. PCR
mutagenesis will also find use for effecting the desired

10 mutations.
        Random mutagenesis of the nucleotide sequences
obtained as described above can be accomplished by
several different techniques known in the art, such as by
altering sequences within restriction endonuclease sites,

15 inserting an oligonucleotide linker randomly into a
plasmid, by irradiation with X-rays or ultraviolet light,
by incorporating incorrect nucleotides during *in vitro*
DNA synthesis, by error-prone PCR mutagenesis, by
preparing synthetic mutants or by damaging plasmid DNA *in*

20 *vitro* with chemicals. Chemical mutagens include, for
example, sodium bisulfite, nitrous acid, hydroxylamine,
agents which damage or remove bases thereby preventing
normal base-pairing such as hydrazine or formic acid,
analogues of nucleotide precursors such as

25 nitrosoguanidine, 5-bromouracil, 2-aminopurine, or
acridine intercalating agents such as proflavine,
acriflavine, quinacrine, and the like. Generally,
plasmid DNA or DNA fragments are treated with chemicals,
transformed into *E. coli* and propagated as a pool or

30 library of mutant plasmids.
        Large populations of random enzyme variants can
be constructed *in vivo* using "recombination-enhanced
mutagenesis" as described in U.S. Patent No. 5,521,077 to
Khosla et al.

35

The gene sequences, native or mutant, which collectively encode PKS protein(s) at least sufficient to catalyze condensation of an extender unit, can be inserted into one or more expression vectors, using
5   methods known to those of skill in the art. In order to incorporate a random assortment of PKS genes, modules, active sites or portions thereof into am expression vector, a cocktail of same can be prepared and used to generate the expression vector by techniques well known
10   in the art and described in detail below. Expression vectors will include control sequences operably linked to the desired PKS coding sequence. Suitable expression systems for use with the present invention include systems which function in eucaryotic and procaryotic host
15   cells. However, as explained above, procaryotic systems are preferred, and in particular, systems compatible with *Streptomyces spp.* are of particular interest. Control elements for use in such systems include promoters, optionally containing operator sequences, and ribosome
20   binding sites. Particularly useful promoters include control sequences derived from PKS gene clusters which result in the production of functional PKS enzymes, such as one or more *act* promoters, *tcm* promoters, spiramycin promoters, and the like. However, other bacterial
25   promoters, such as those derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) and maltose, will also find use in the present constructs. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*), the
30   β-lactamase (*bla*) promoter system, bacteriophage lambda PL, and T5. In addition, synthetic promoters, such as the *tac* promoter (U.S. Patent No. 4,551,433), which do not occur in nature also function in bacterial host cells.

35

Other regulatory sequences may also be
desirable which allow for regulation of expression of the
PKS genes relative to the growth of the host cell.
Regulatory sequences are known to those of skill in the

5   art, and examples include those which cause the
expression of a gene to be turned on or off in response
to a chemical or physical stimulus, including the
presence of a regulatory compound. Other types of
regulatory elements may also be present in the vector,

10   for example, enhancer sequences.

       Selectable markers can also be included in the
recombinant expression vectors. A variety of markers are
known which are useful in selecting for transformed cell
lines and generally comprise a gene whose expression

15   confers a selectable phenotype on transformed cells when
the cells are grown in an appropriate selective medium.
Such markers include, for example, genes which confer
antibiotic resistance or sensitivity to the plasmid.
Alternatively, several polyketides are naturally colored

20   and this characteristic provides a built-in marker for
selecting cells successfully transformed by the present
constructs, i.e., that express a functional PKS that can
be isolated and used to catalytically prepare polyketides
in a cell-free system.

25   The various PKS subunits of interest, or the
cocktail of PKS genes, modules, active sites, or portions
thereof, can be cloned into one or more recombinant
vectors as individual cassettes, with separate control
elements, or under the control of, e.g., a single

30   promoter. The PKS subunits or cocktail components can
include flanking restriction sites to allow for the easy
deletion and insertion of other PKS subunits or cocktail
components so that hybrid PKSs can be generated. The
design of such unique restriction sites is known to those

35   of skill in the art and can be accomplished using the

techniques described above, such as site-directed
mutagenesis and PCR.

Using these techniques plasmid pRM5 was
constructed as a shuttle vector for the production of the
5   PKS enzymes for use in a cell-free system described
herein. Plasmid pRM5 includes the genes encoding the
actinorhodin PKS subunits flanked by *PacI* and *NsiI*
restriction sites. A new nucleotide sequence encoding a
PKS flanked by *PacI* and *NsiI* sites can be easily
10   substituted for the actinrhodin PKS genes. The shuttle
plasmid also contains the *act* KR gene (*actIII*), the
cyclase gene (*actVII*), and a putative dehydratase gene
(*actIV*), as well as a *ColEI* replicon (to allow
transformation of *E. coli*), an appropriately truncated
15   SCP2* (low copy number) *Streptomyces* replicon, and the
actII-ORF4 activator gene from the *act* cluster, which
induces transcription from *act* promoters during the
transition from growth phase to stationary phase in the
vegetative mycelium. pRM5 carries the divergent
20   *actI/actIII* promoter pair.

Methods for introducing the recombinant vectors
of the present invention into suitable hosts are known to
those of skill in the art and typically include the use
of $CaCl_2$ or other agents, such as divalent cations and
25   DMSO. DNA can also be introduced into bacterial cells by
electroporation.

The cells modified to contain expression
systems for functional PKS proteins are then cultured
under conditions wherein these proteins are produced.

30

2.   Preparation of the Cell-Free System

If the polyketide synthase proteins for use in
the cell-free system are to be prepared recombinantly as
described above, the cells producing the relevant PKS
35   proteins are optionally harvested and disrupted if the

desired proteins have been intracellularly produced.
However, if the expression system secretes the protein
into growth media, the protein can be purified directly
from the media.

5   If the protein is not secreted, it can be
isolated from cell lysates. This is generally
accomplished by first preparing a crude extract which
lacks cellular components and several extraneous
proteins. The desired proteins can then be further
10   purified i.e. by column chromatography, HPLC,
immunoadsorbent techniques or other conventional methods
well known in the art. The selection of the appropriate
growth conditions and recovery methods are within the
skill of the art.

15   For example, cells that express the PKS of
interest can be grown to produce a predetermined number
of cells. The cells may be disrupted by sonication,
freeze-thaw cycles or other like techniques by which the
cell membrane is breached to form a crude cell-free
20   preparation. The crude cell-free preparation may be used
at this stage as a source of PKS or may be further
processed by centrifugation, filtration or the like, to
form a cell supernatant. Optionally, nucleic acids may
be removed from the cell supernatant by, for example,
25   precipitation with polyethyleneimine, or other like agent
which does not disturb the enzymatic activity of the PKS.
The preparation may be used at this stage as a source of
PKS. Optionally, the PKS may be further purified by
techniques known to those of skill in the art.

30   For use in the construction of libraries of
polyketides, in addition to recombinantly produced
polyketide synthase proteins, isolated native forms may
in some instances be used.

          The purified PKS can be used to catalytically
35   synthesize polyketides in a cell-free system as

exemplified below. The cell-free system includes purified PKS, in an appropriate buffer, and the substrates required for the catalytic synthesis of polyketides. Depending on the PKS, starter substrate

5   units can include, e.g., acetyl-CoA, malonamyl-CoA, propionyl-CoA, butyryl-CoA, isobutyryl-CoA, isovaleryl-CoA, aromatic coenzyme A thioesters such as benzoyl-CoA, aminobenzoyl-CoA, aminohydroxy benzoyl-CoA, and the like, heterocyclics such as thiophenecarboxyl-CoA, and the

10   like, and partially synthesized polyketides. Alternatively, the coenzyme A thioesters may be replaced by corresponding N-acetylcysteamine thioesters. Extender units include, for example, malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA, and other like molecules well

15   known to those of skill in the art.

It has not been possible, heretofore, to provide cell-free systems for the synthesis of polyketides using isolated or purified modular polyketide synthases. According to the present invention, such

20   cell-free systems are provided even for the production of polyketides produced by these complex synthases.

The polyketides that are prepared using the cell-free system disclosed herein may be isolated and identified using any of a variety of techniques known in

25   the art (see, e.g., WO 95/08548) including thin layer chromatography, high performance liquid chromatography, analytical and/or preparative gel electrophoresis, column chromatography, gas chromatography, nuclear magnetic resonance ("NMR"), mass spectrometry, or other

30   conventional methods well known in the art.

        3.   <u>Additional Background Information on PKS</u>
<u>and Use in Library Design</u>

The cell-free preparations described above are

35   particularly useful in constructing polyketide libraries

that contain a multiplicity of different polyketides. It
will be useful to review the variations that can be
included by virtue of varying the proteins containing the
PKS catalytic activities required for synthesis.

5 Although hybrid systems can be obtained which combine
coding sequences derived from aromatic and modular and
fungal PKS, it may be helpful to describe in more detail
the mode of action of these PKSs and the combinatorial
possibilities. For ease of explanation, the aromatic,

10 modular and fungal PKS systems are discussed separately.

Generally, polyketide synthesis occurs in three
stages. In the first stage, catalyzed by the PKS, a
nascent polyketide backbone is generated from monomeric
CoA thioesters. In the second stage this backbone is

15 regiospecifically cyclized. While some cyclization
reactions are controlled by the PKS itself, others result
from activities of downstream enzymes. In the final
stage, the cyclized intermediate is modified further by
the action of mechanistically diverse "tailoring

20 enzymes," giving rise to the natural product.

a)   Aromatic PKS

Background: For aromatic PKS, polyketide
biosynthesis begins with a primer unit loading on to the

25 active site of the condensing enzyme, β-keto acyl
synthase/acyl transferase (KS/AT). An extender unit
(usually malonate) is then transferred to the
pantetheinyl arm of the acyl carrier protein (ACP). The
KS/AT catalyzes the condensation between the ACP-bound

30 malonate and the starter unit. Additional extender units
are added sequentially until the nascent polyketide chain
has grown to a desired chain length determined by the
protein chain length factor (CLF), perhaps together with
the KS/AT. Thus, the KS, CLF and the ACP form a minimal

35 set to generate a polyketide backbone. The nascent

polyketide chain is then subjected to regiospecific
ketoreduction by a ketoreductase (KR) if it exists.
Cyclases (CYC) and aromatases (ARO) later catalyze
regiospecific ring formation events through

5   intramolecular aldol condensations. The cyclized
intermediate may then undergo additional regiospecific
and/or stereospecific modifications (e.g., O-methylation,
hydroxylation, glycosylation, etc.) controlled by
downstream tailoring enzymes).

10   Acetyl-CoA is the usual starter unit for most
aromatic polyketides. However, malonamyl-CoA (Gatenbeck,
S. *Biochem. Biophy. Res. Commun.* (1961) $\underline{6}$:422-426) and
propionyl-CoA (Paulick, R. C. *et al. J. Am. Chem. Soc.*
(1976) $\underline{98}$:3370-3371) are primers for many members of the

15   tetracycline and anthracycline classes of polyketides,
respectively. Daunorubicin PKS can also accept acetyl-
CoA, butyryl-CoA, and isobutyryl-CoA as starter units.
(Oki, T. *et al. J. Antibiot.* (1981) $\underline{34}$:783-790;
Yoshimoto, A. *et al. J. Antibiot.* (1993) $\underline{46}$:1758-1761).

20   The *act* KR can productively interact with all
minimal PKSs studied thus far and is both necessary and
sufficient to catalyze a C-9 ketoreduction. Although
homologous KRs have been found in other PKS clusters,
they catalyze ketoreduction with the same

25   regiospecificity. However, the structures of frenolicin,
griseusin and daunorubicin suggest that an additional
C-17 ketoreduction occurs in these biosynthetic pathways.
Likewise, several angucyclines undergo a C-15
ketoreduction, which occurs before the nascent polyketide

30   chain is cyclized (Gould, S. J. *et al. J. Am. Chem. Soc.*
(1992) $\underline{114}$:10066-10068). The ketoreductases responsible
for C-15 and C-17 reductions have not yet been
identified; however, two homologous KRs have been found
in the daunorubicin PKS cluster (Grimm, A. *et al. Gene*

35   (1994) $\underline{151}$:1-10; Ye, J. *et al. J. Bacteriol.* (1994)

176:6270-6280). It is likely that they catalyze the C-9 and C-17 reductions.

The formation of the first two six-membered rings in the biosynthesis of most naturally occurring bacterial aromatic polyketides is controlled by PKS subunits; further ring closures are controlled by additional cyclases and modifying enzymes. The structural diversity introduced via these reactions appears to be greater than via the first two cyclizations. However, certain preferred patterns are observed, which suggests that at least some of these downstream cyclases may be useful for the construction of combinatorial libraries. For example, the pyran ring in isochromanequinones is invariably formed via cyclization between C-3 and C-15; two stereochemically distinct classes of products are observed. In anthracyclines and tetracyclines a third aldol condensation usually occurs between C-3 and C-16, whereas in unreduced tetracenomycins and related compounds it occurs between C-5 and C-18, and in angucyclines it occurs between C-4 and C-17. Representative gene(s) encoding a few of these enzymes have already been cloned (Fernandez-Moreno, M. A. et al. J. Biol. Chem. (1994) 269:24854-24863; Shen, B. et al. Biochemistry (1993) 32:11149-11154). At least some cyclases might recognize chains of altered lengths and/or degrees of reduction, thereby increasing the diversity of aromatic polyketide combinatorial libraries.

In the absence of downstream cyclases, polyketide chains undergo non-enzymatic reactions. Recently, some degree of predictability has emerged within this repertoire of possibilities. For instance, hemiketals and benzene rings are two common moieties seen on the methyl end. Hemiketals are formed with an appropriately positioned enol and can be followed by a dehydration. Benzene rings are formed with longer

uncyclized methyl terminus. On the carboxyl terminus, a
γ-pyrone ring formed by three ketide units is frequently
observed. Spontaneous decarboxylations occur on free
carboxyl ends activated by the existence of a β-carbonyl.

5   A cyclized intermediate can undergo various
types of modifications to generate the final natural
product. The recurrence of certain structural motifs
among naturally occurring aromatic polyketides suggests
that some tailoring enzymes, particularly group

10   transferases, may be combinatorially useful. Two
examples are discussed below.

O-methylation is a common downstream
modification. Although several SAM-dependent
O-methyltransferase genes have been found in PKS gene

15   clusters (Decker, H. *et al. J. Bacteriol.* (1993)
175:3876-3886), their specificities have not been
systematically studied as yet. Perhaps some of them
could be useful for combinatorial biosynthesis. For
instance, O-11-methylation occurs in several members of

20   the anthracycline, tetracenomycin, and angucycline
classes of aromatic polyketides.

Library Design: The following set of design
rules permits rationally or stochastically manipulating

25   early biosynthetic steps in aromatic polyketide pathways
including chain synthesis, C-9 ketoreduction, and the
formation of the first two aromatic rings. If each
biosynthetic degree of freedom is independent of all
others, then it should be possible to design a single

30   combinatorial library of $N_1 \times N_2 \times \cdots N_i \times \cdots N_{n-1} \times N_n$
clones, where N_i is the number of ways in which the ith
degree of freedom can be exploited. In practice however,
not all enzymatic degrees of freedom are independent.
Therefore, to minimize redundancy, it is preferable to

35

design several sub-libraries of PKS enzyme-producing
clones.

(1) Chain length. In the aromatic synthases,
polyketide carbon chain length is dictated by the minimal
5   PKS. Within the minimal PKS, the acyl carrier protein
can be interchanged without affecting specificity,
whereas the chain length factor is crucial. Although
some ketosynthase/chain length factor combinations are
functional, others are not; therefore, biosynthesis of a
10   polyketide chain of specified length can be insured with
a minimal PKS in which both the ketosynthase and chain
length factor originate from the same PKS gene cluster.
So far, chain lengths of 16 (octaketide), 18
(nonaketide), 20 (decaketide), and 24 carbons
15   (dodecaketide) can be generated with minimal PKSs from
the *act, fren, tcm*, and, *whiE* PKS clusters, respectively
(McDaniel *et al. Science* (1993), *supra*; McDaniel *et al.*
J. Am. Chem. Soc. (1993), *supra*; McDaniel *et al. Proc.*
Natl. Acad. Sci. USA (1994), *supra*). The *whiE* minimal
20   PKS can also generate 22-carbon backbones in the presence
of a KR, suggesting a degree of relaxed chain length
control as found for the *fren* PKS.

(2) Ketoreduction. Ketoreduction requires a
ketoreductase. The *act* KR can catalyze reduction of the
25   C-9 carbonyl (counting from the carboxyl end) of a
nascent polyketide backbone of any length studied so far.
Furthermore, the *act* KR is compatible with all the
minimal PKSs mentioned above. Homologous ketoreductases
have been identified in other PKS clusters (Sherman,
30   D.H., *et al. EMBO J.* (1989) 8:2717-2725; Yu, T.-W. *et al.*
J. Bacteriol. (1994) 176:2627-2534; Bibb, M.J. *et al.*
Gene (1994) 142:31-39). These enzymes may catalyze
ketoreduction at C-9 as well since all the corresponding
natural products undergo this modification. In unusual

35

circumstances, C-7 ketoreductions have also been observed
with the *act* KR.

   (3) Cyclization of the first ring. Although
the minimal PKS alone can control formation of the first
ring, the regiospecific course of this reaction may be
influenced by other PKS proteins. For example, most
minimal PKSs studied so far produce polyketides with
C-7/C-12 cyclizations when present alone. In contrast,
the *tcm* minimal PKS alone generates both C-7/C-12 and
C-9/C-14 cyclized products. The presence of a
ketoreductase with any minimal PKS restricts the nascent
polyketide chain to cyclize exclusively with respect to
the position of ketoreduction: C-7/C-12 cyclization for
C-9 ketoreduction and C-5/C-10 cyclization for C-7
ketoreduction (McDaniel, R. *et al. J. Am. Chem. Soc.*
(1993) 115:11671-11675; McDaniel, R. *et al. Proc. Natl.*
Acad. Sci. USA (1994) 91:11542-11546; McDaniel, R. *et al.*
J. Am. Chem. Soc. (1994) 116:1085510859). Likewise, use
of the TcmN enzyme alters the regiospecificity to
C-9/C-14 cyclizations for unreduced polyketides of
different lengths, but has no effect on reduced
molecules.

   (4) First ring aromatization. The first ring
in unreduced polyketides aromatizes non-catalytically.
In contrast, an aromatizing subunit is required for
reduced polyketides. There appears to be a hierarchy in
the chain length specificity of these subunits from
different PKS clusters. For example, the *act* ARO will
recognize only 16-carbon chains (McDaniel *et al. Proc.*
Natl. Acad. Sci. USA (1994), *supra*), the *fren* ARO
recognizes both 16- and 18-carbon chains, while the *gris*
ARO recognizes chains of 16, 18, and 20 carbons.

   (5) Second ring cyclization. C-5/C-14
cyclization of the second ring of reduced polyketides may
be achieved with an appropriate cyclase. While the *act*

CYC can cyclize octa- and nonaketides, it does not
recognize longer chains. No equivalent C-5/C-14 cyclase
with specificity for decaketides or longer chains has
been identified, although the structures of natural

5   products such as griseusin imply their existence. In the
case of sufficiently long unreduced chains with a
C-9/C-14 first ring, formation of a C-7/C-16 second ring
is catalyzed by the minimal PKS (McDaniel et al. Proc.
Natl. Acad. Sci. USA (1994), supra).

10   (6) Additional cyclizations. The KS/AT, CLF,
ACP, KR, ARO, and CYC subunits of the PKS together
catalyze the formation of an intermediate with a defined
chain length, reduction pattern, and first two
cyclizations. While the biosynthesis of naturally

15   occurring polyketides typically requires the activity of
downstream cyclases and other modifying enzymes to
generate the characteristic biologically active product,
subsequent reactions in the biosynthesis of engineered
polyketides described here and in our earlier work occur

20   in the absence of specific enzymes and are determined by
the different physical and chemical properties of the
individual molecules. Presumably reflecting such
chemical possibilities and constraints, consistent
patterns have been observed, leading to some degree of

25   predictability. Two common moieties formed by the
uncyclized methyl terminus of polyketide chains are
hemiketals and benzene rings. Formation of a hemiketal
occurs in the presence of an appropriately positioned
enol and can be followed by a dehydration since both the

30   hydrated and dehydrated forms are often isolated
(McDaniel, R. et al. Science (1993) 262:15461550;
McDaniel, R. et al. J. Am. Chem. Soc. (1994)
116:1085510859; Fu, H. et al. J. Am. Chem. Soc. (1994)
116:41664170), while benzene ring formation occurs with

35   longer unprocessed methyl ends (Fu et al. J. Am. Chem.

Soc. (1994), *supra*). The most frequently observed moiety at the carboxyl terminus of the chain is a γ-pyrone ring formed by three ketide units (McDaniel *et al. J. Am. Chem. Soc.* (1994), *supra*; Fu *et al. J. Am. Chem. Soc.*

5   (1994), *supra*; Fu, H., *et al. Biochemistry* (1994)
33:9321-9326; Fu, H. *et al. Chem. & Biol.* (1994)
1:205-210; Zhang, H.-1. *et al. J. Org. Chem.* (1990)
55:1682-1684); if a free carboxylic acid remains,
decarboxylation typically occurs if a β-carbonyl exists

10   (McDaniel *et al. Science* (1993), *supra*; McDaniel, R.,
Ebert-Khosla, S., Hopwood, D.A. & Khosla, C. *J. Am. Chem. Soc.* (1993), *supra*; Kao, C.M. *et al. J. Am. Chem. Soc.*
(1994) 116:11612-11613). Many aldol condensations can be
predicted as well, bearing in mind that the methyl and

15   carboxyl ends tend preferentially to cyclize
independently but will co-cyclize if no alternative
exists (McDaniel *et al. Proc. Natl. Acad. Sci. USA*
(1994), *supra*. These non-enzymatic cyclization patterns
observed *in vivo* are also consistent with earlier

20   biomimetic studies (Griffin, D.A. *et al. J. Chem. Soc. Perkin Trans.* (1984) 1:1035-1042).

Taken together with the structures of other naturally occurring bacterial aromatic polyketides, the design rules presented above can be extrapolated to

25   estimate the extent of molecular diversity that might be generated via *in vivo* combinatorial biosynthesis of, for example, reduced and unreduced polyketides. For reduced polyketides, the identified degrees of freedom include chain length, aromatization of the first ring, and

30   cyclization of the second ring. For unreduced ones, these include chain length and regiospecificity of the first ring cyclization. The number of accessible structures is the product of the number of ways in which each degree of freedom can be varied. Chains of five

35   different lengths have so far been manipulated (16-, 18-

generated from combinatorial manipulation of only the first few steps in aromatic polyketide biosynthesis is on the order of a few hundred. Thus, genetically engineered biosynthesis represents a potentially unlimited source of

5 chemical diversity for drug discovery.

   b) Modular PKS

      Background: As illustrative of synthesis by modular PKS, polyketide biosynthesis by DEBS begins with

10 the first acyltransferase (AT) activity in module 1 loading the starter unit onto the module 1 condensing activity, the β-ketoacylsynthase (KS). The second AT of module 1 loads the first extender unit onto the pantetheinyl arm of the acyl-carrier protein (ACP)

15 activity. The KS catalyzes the decarboxylative condensation between the ACP-bound malonyl unit and the primer unit. The resulting diketide is then reduced by the ketoreductase (KR) activity of module 1, which converts the β-keto group into an alcohol. In more

20 complex modules, such as DEBS module 4, additional reductive cycle activities (a dehydratase (DH) and an enoylreductase (ER)) come into play after the module's KR performs the initial ketoreduction. The module 1 product is then passed to the module 2 KS. The module 2 AT loads

25 the second extender unit onto the module 2 ACP, and the module 2 KS then performs the condensation to produce a triketide which is reductively processed. Additional modules come into play, each adding and processing another extender unit onto the growing polyketide chain.

30 The final length of the polyketide chain is determined by the number of modules present in the PKS (six in the case of DEBS), and the reductive outcome at any position is determined by the complement of reductive cycle activities present in the corresponding module. After

35. elaboration of the polyketide chain, the molecule is

subjected to regiospecific cyclization by a thioesterase
(TE) activity fused to the end of DEBS module 6.   The
macrolide product is then tailored by downstream enzymes,
e.g., hydroxylases, oxidases, methyltransferases,

5   glycosylases, and the like, to produce the final natural
product.

Library Construction: The following set of
design rules applies for rationally or stochastically

10   manipulating early biosynthetic steps in modular
polyketide biosynthetic pathways.   The manipulative
elements include:

(1) Starter Unit.   The relaxed specificity of
modular PKSs for the starter unit under *in vitro*

15   conditions has been reported (Pieper *et al*. 1995,
supra).

(2) Extender Unit.   The nature of the extender
unit used by a given module is determined by the AT
activity.   Sequence comparisons have clearly identified

20   the characteristics of malonyl-CoA-specific AT and
methylmalonyl-CoA-specific AT activities (Haydock *et al*.
1995, *supra*) At activities using methylmalonyl-CoA or
ethylmalonyl-CoA generate a chiral center having one of
two possible stereochemistries.   For the two common

25   extender units, malonyl-CoA and methylmalonyl-CoA, there
are thus three possible structural outcomes.

(3) Reductive Cycle.   The state of reduction of
the β-keto group formed by KS-catalyzed condensation is
governed by the set of reductive cycle activities present

30   within the module.   Thus, absence of any reductive
activity yields a ketone function, while presence of only
a KR activity generates an alcohol group having one of
two possible stereochemistries.   The presence of both KR
and DH activities results in the formation of an alkene;

35   if a stereocenter had been generated by the AT activity,

the chirality at that position is lost. The presence of
the full complement of KR, DH, and ER activities results
in complete reduction of the β-keto group to a methylene
group. There are thus 5 theoretically possible reductive
5   cycle outcomes at any module.

(4) Cyclizations. The linear polyketide chain
may cyclize through a number of possible mechanisms. The
DEBS thioesterase (TE) activity demonstrates a broad
capacity to lactonize hydroxy-acids (Aggarwal *et al.*
10   1995, *supra*). Also, several known natural products,
e.g., avermectin, mevinolin, appear to be formed through
Diels-Alder cyclizations of polyketide chains containing
multiple alkene groups. Cyclizations of alcohols onto
ketones to form ketals and spiroketals is also commonly
15   observed.

For any single module, therefore, there are at
least 14 theoretical structural outcomes when only the
two common extender units are considered. If all
manipulable elements can be simultaneously controlled,
20   there are

$$S \times (14)^N$$

possible polyketide chains which can be produced from an
N-module PKS using S starter units. For a 6-module PKS
such as DEBS, 14^6, or more than 7.5×10^6 polyketide
25   chains could be produced using a single starter unit.
Furthermore, enzymes that catalyze downstream
modifications, e.g., cyclizations, group-transfer
reactions, oxidoreductions, and the like, can be studied
along the lines presented herein and elsewhere. It is
30   therefore possible that at least some of these degrees of
freedom can be combinatorially exploited to generate
libraries of synthetic products with structural diversity
that is comparable to that observed in nature.

Although modular PKSs have not been extensively
35   analyzed, the one-to-one correspondence between active

sites and product structure, together with the incredible
chemical diversity observed among naturally occurring
"complex" polyketides, indicates that the combinatorial
potential within these multienzyme systems could be

5   considerably greater than that for aromatic PKSs. For
example, a wider range of primer units including
aliphatic monomers (acetate, propionate, butyrate,
isovalerate, etc.), aromatics (aminohydroxybenzoic acid),
alicyclics (cyclohexanoic acid), and heterocyclics

10   (pipecolic acid) are found in various macrocyclic
polyketides. Recent studies have shown that modular PKSs
have relaxed specificity for their starter units (Kao *et
al. Science* (1994), *supra*). The degree of
β-ketoreduction following a condensation reaction can

15   also be altered by genetic manipulation (Donadio *et al.
Science* (1991), *supra*; Donadio, S. *et al. Proc. Natl.
Acad. Sci. USA* (1993) 90:7119-7123). Likewise, the size
of the polyketide product can be varied by designing
mutants with the appropriate number of modules (Kao, C.M.

20   *et al. J. Am. Chem. Soc.* (1994) 116:11612-11613).
Modular PKSs also exhibit considerable variety with
regards to the choice of extender units in each
condensation cycle, although it remains to be seen to
what extent this property can be manipulated. Lastly,

25   these enzymes are particularly well-known for generating
an extensive range of asymmetric centers in their
products in a highly controlled manner. Thus, the
combinatorial potential within modular PKS pathways could
be virtually unlimited.

30

       c)   Glycosylation
       Both aromatic and complex polyketides are often
glycosylated. In many cases (e.g., doxorubicin and
erythromycin) absence of the sugar group(s) results in

35   considerably weaker bioactivity. There is tremendous

diversity in both the types and numbers of sugar units attached to naturally occurring polyketide aglycones. In particular, deoxy- and aminosugars are commonly found. Regiochemical preferences can be detected in many

5   glycosylated natural products. Among anthracyclines, O-17 is frequently glycosylated, whereas among angucyclines, C-10 is usually glycosylated. Glycosyltransferases involved in erythromycin biosynthesis may have relaxed specificities for the

10   aglycone moiety (Donadio, S. et al. Science (1991) 252:675-679). An elloramycin glycosyltransferase may be able to recognize an unnatural NDP-sugar unit and attach it regiospecifically to an aromatic polyketide aglycone (Decker, H. et al. Angew. Chem. (1995), in press). These

15   early results suggest that glycosyltransferases derived from secondary metabolic pathways have unique properties and may be attractive targets for use in the generation of combinatorial libraries.

20   d)   Fungal PKS
            Like the actinomycetes, filamentous fungi are a rich source of polyketide natural products. The fact that fungal PKSs, such as the 6-methylsalicylic acid synthase (6-MSAS) and the mevinolin synthase, are encoded by

25   single multi-domain proteins (Beck et al. Eur. J. Biochem. (1990), supra; Davis, R. et al. Abstr. Genet. Ind. Microorg. Meeting, supra) indicates that they may also be targeted for combinatorial mutagenesis. Moreover, fungal PKSs can be functionally expressed in S.

30   coelicolor CH999 using the genetic strategy outlined above and described in WO 95/08548, supra. Chain lengths not observed in bacterial aromatic polyketides (e.g., tetraketides, pentaketides and hexaketides) have been found among fungal aromatic polyketides (O'Hagan, D. The

35   Polyketide Metabolites (Ellis Horwood, Chichester, U.K.,

1991). Likewise, the cyclization patterns of fungal
aromatic polyketides are quite different from those
observed in bacterial aromatic polyketides (*Id*.). In
contrast with modular PKSs from bacteria, branched methyl

5   groups are introduced into fungal polyketide backbones by
S-adenosylmethionine-dependent methyltransferases; in the
case of the mevinolin PKS (Davis, R. *et al*. *Abstr. Genet.*
Ind. Microorg. Meeting, supra), this activity is encoded
as one domain within a monocistronic PKS. It is now

10   possible to experimentally evaluate whether these and
other sources of chemical diversity in fungal polyketides
are indeed amenable to combinatorial manipulation.
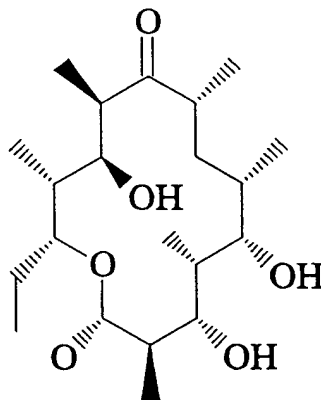

     e)   Summary:

15   The number of potentially novel polyketides
that can be catalytically produced by PKS gene products
in a cell-free system increases geometrically as new
degrees of freedom are exploited and/or protein
engineering strategies are brought to bear on the task of

20   creating enzyme subunits with specificities not observed
in nature. For example, non-acetate starter units can be
incorporated into polyketide backbones (e.g., propionate
in daunorubicin and malonamide in oxytetracycline).
Furthermore, enzymes that catalyze downstream

25   cyclizations and late-step modifications, such as group
transfer reactions and oxidoreductions commonly seen in
naturally occurring polyketides, can be studied along the
lines presented here and elsewhere. It is therefore
possible that at least some of these degrees of freedom

30   can be combinatorially exploited to generate libraries of
synthetic products with structural diversity that is
comparable to that observed in nature.

35

C.   Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not

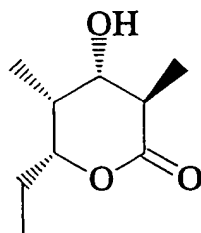5   intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should,

10   of course, be allowed for.

The Examples provided below describe recombinant production of a modular PKS and methods for *in vitro* synthesis of polyketides by recombinant DEBS and by an active deletion mutant. The latter mutant,

15   designated "DEBS 1+2+TE", contains the first two modules from DEBS 1 fused to the thioesterase domain normally found at the C-terminal end of module 6 of DEBS (Figure 1). Both DEBS and DEBS 1+2+TE have been successfully expressed in *S. coelicolor* CH999, purified, and used in a

20   cell-free system for the *in vitro* catalytic synthesis of, respectively, 6-dEB (**1**)

(**1**)

and (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2**)

35

$$OH$$

(2).

Three open reading frames (*eryAI, eryAII,* and *eryAIII*) encode the DEBS polypeptides and span 32 kb in the *ery* gene cluster of the *Saccharopolyspora erythraea* genome. The genes are organized in six repeated units, each designated a "module" (*see* Figure 1). Each module encodes a set of active sites that, during polyketide biosynthesis, catalyzes the condensation of an additional monomer onto the growing chain. Each module includes an acyltransferase (AT), β-ketoacyl carrier protein synthase (KS), and acyl carrier protein (ACP) as well as a subset of reductive active sites (β-ketoreductase (KR), dehydratase (DH), enoyl reductase (ER)). The number of reductive sites within a module corresponds to the extent of β-keto reduction in each condensation cycle. The thioesterase (TE) encoded at the end of module appears to catalyze lactone formation.

Due to the large sizes of *eryAI, eryAII,* and *eryAIII,* and the presence of multiple active sites, these genes can be conveniently cloned into a plasmid suitable for expression in a host cell, such as the genetically engineered host cell CH999, using an *in vivo* recombination technique. This technique, described in WO 95/08548 utilizes derivatives of the plasmid pMAK705 (Hamilton *et al.* (1989) *J. Bacteriol.* 171:4617) to permit *in vivo* recombination between a temperature-sensitive donor plasmid, which is capable of replication at a first, permissive temperature and incapable of replication at a second, non-permissive temperature, and

recipient plasmid. The *eryA* genes thus cloned gave pCK7, a derivative of pRM5 (McDaniel *et al.* (1993) *Science* 262:1546). A control plasmid, pCK7f, was constructed to carry a frameshift mutation in *eryAI*. pCK7 and pCK7f

5   possess a *ColEI* replicon for genetic manipulation in *E. coli* as well as a truncated SCP2* (low copy number) *Streptomyces* replicon. These plasmids also contain the divergent *actI/actIII* promoter pair and *actII-ORF4*, an activator gene, which is required for transcription from

10 these promoters and activates expression during the transition from growth to stationary phase in the vegetative mycelium. High-level expression of PKS genes occurs at the onset of stationary phase of mycelial growth.

15

Example 1
Production of *S. coelicolor* CH999

An *S. coelicolor* host cell, genetically engineered to remove the native *act* gene cluster, and

20 termed CH999, was constructed using *S. coelicolor* CH1 (Khosla *et al.* *Molec. Microbiol.* (1992), *supra*) as described in WO95/08548, incorporated herein by reference. *S. coelicolor* CH999 lacks the entire ACT gene cluster.

25

Example 2
Production of the Recombinant Vector pRM5

Shuttle plasmids are used to express recombinant PKSs in CH999. Such plasmids typically

30 include a colEI replicon, an appropriately truncated SCP2* *Streptomyces* replicon, two *act*-promoters to allow for bidirectional cloning, the gene encoding the actII-ORF4 activator which induces transcription from *act* promoters during the transition from growth phase to

35 stationary phase, and appropriate marker genes.

Restriction sites have been engineered into these vectors
to facilitate the combinatorial construction of PKS gene
clusters starting from cassettes encoding individual
subunits (or domains) of naturally occurring PKSs. Among

5   the many advantages of this method are that (i) all
relevant biosynthetic genes are plasmid-borne and
therefore amenable to facile manipulation and mutagenesis
in *E. coli* and (ii) the entire library of PKS gene
clusters can be expressed in the same bacterial host.

10   pRM5 was the shuttle plasmid used for
expressing PKSs in CH999. It includes a *ColEI* replicon
to allow genetic engineering in *E. coli*, an appropriately
truncated SCP2* (low copy number) *Streptomyces* replicon,
and the *actII-ORF4* activator gene from the *act* cluster,

15   which induces transcription from *act* promoters during the
transition from growth phase to stationary phase in the
vegetative mycelium. pRM5 carries the divergent
actI/actIII promoter pair, together with convenient
cloning sites to facilitate the insertion of a variety of

20   engineered PKS genes downstream of both promoters. pRM5
lacks the par locus of SCP2*; as a result the plasmid is
slightly unstable (approx. 2% loss in the absence of
thiostrepton). This feature was deliberately introduced
in order to allow for rapid confirmation that a phenotype

25   of interest could be unambiguously assigned to the
plasmid-borne mutant PKS. The recombinant PKSs from pRM5
are expressed approximately at the transition from
exponential to stationary phase of growth, in good
yields.

30   pRM5 was constructed as described in
WO95/08548.

35

Example 3
Construction of Expression Vectors
for, and Expression of Aromatic PKS

WO95/08548 describes the construction of

5   expression vectors using the pRM5 host plasmid using
portions of the aromatic polyketide synthase gene
clusters of actinorhodin (act), granaticin (gra) and
tetracenomycin (tcm) gene clusters. A number of hybrid
clusters are described. These hybrid clusters were

10   introduced into S. coelicolor CH999 and expressed to
produce the relevant polyketide synthases which in turn
produce a variety of polyketides. Additional constructs
using genes derived from the frenolicin B (fren) PKS gene
cluster were also prepared.

15

Example 4
Production of Modular PKS

Expression plasmids containing recombinant
modular DEBS PKS genes were constructed by transferring

20   DNA incrementally from a temperature-sensitive "donor"
plasmid, i.e., a plasmid capable of replication at a
first, permissive temperature and incapable of
replication at a second, non-permissive temperature, to a
"recipient" shuttle vector via a double recombination

25   event, as described in WO95/08548, and in Kai et al.
Science (1994) 265:509. pCK7, contains the complete eryA
gene. A control plasmid, pCK7f, which contains a
frameshift error in eryAI, was constructed in a similar
manner. pCK7 and pCK7f were transformed into E. coli

30   ET12567 (MacNeil (1988) J. Bacteriol. 170:5607) to
generate unmethylated plasmid DNA and subsequently moved
into S. coelicolor CH999 using standard protocols
(Hopwood et al. (1985) Genetic manipulation of
Streptomyces. A laboratory manual. The John Innes

35   Foundation: Norwich).

Upon growth of CH999/pCK7 on R2YE medium, two polyketides were produced. In addition, three high-molecular-weight proteins (>200 kDa) presumably DEBS1, DEBS2 and DEBS3 (Caffrey *et al.* *FEBS Lett.* (1992)

5   304:225) were also observed in crude extracts of CH999/pCK7 via sodium dodecyl sulfate-polyacrylamide gel electrophoresis ("SDS-PAGE"). No polyketide products were observed from CH999/pCK7F.

10   Example 5
       Recombinant Production of a Mutant DEBS PKS
            In this Example a deletion mutant PKS was constructed that consists of DEBS1 fused to the TE of DEBS3 ("DEBS 1+2+TE"); plasmid pCK12 contained the genes

15   encoding the DEBS 1+2+TE.
            The DEBS 1+2+TE PKS contained a fusion of the carboxy-terminal end of the acyl carrier protein of module 2 (ACP-2) to the carboxy-terminal end of the acyl carrier protein of module 6 (ACP-6) (*see* Figure 1). Thus

20   ACP-2 is essentially intact in this PKS and is followed by the amino acid sequence naturally found between ACP-6 and the TE. pCK12 is identical to pCK7 (Kao *et al.* *Science* (1994), *supra*) with the exception of a deletion between the carboxy-terminal ends of ACP-2 and ACP-6.

25   The fusion occurs between residues L3455 of DEBS1 and Q2891 of DEBS3. An *Spe*I site is present between these two residues so that the DNA sequence at the fusion is CTCACTAGTCAG.

30   Example 6
       Preparation of Cell-Free DEBS from pCK7 and pCK12
            The DEBS preparation was carried out as follows. *S. coelicolor* CH999/pCK12 or CH999/pCK7 cells were harvested after a growth of 55 h in liquid cultures.

35   Typically, 8-10 grams of cells (wet cell weight) were

disrupted using sonication (5 X 30 s bursts). The
resultant cell slurry was ultracentrifuged (2 h at
192,000 x g) and nucleic acids precipitated with 0.2%
polyethyleneimine (Step 1) yielding about 200 mg total

5 protein. All 3 DEBS proteins were precipitated in a 55%
saturated ammonium sulfate solution. The incubation
buffer (buffer I) used thereafter contained 150 mM sodium
phosphate buffer (pH 7.1), 15% glycerol, 2 mM
dithiothreitol ("DTT"), and 2 mM ethylene diamine

10 tetraacetic acid ("EDTA"). After desalting on Sephadex
G25 M (Step 2), about 30 mg protein (15-20 mg/mL) was
applied to an Agarose BioGel A size exclusion column (140
mL). Fractions containing DEBS proteins were pooled and
concentrated to 1 mg/mL on YM 30 ultrafiltration

15 membranes (Step 3).

DEBS proteins were detected by their high
molecular weights of 330 kDa (DEBS 3), 370 kDa (DEBS 1)
and 380 kDa (DEBS 2) by SDS-PAGE; these proteins were
absent in cell extracts of a variety of control strains.

20 The apparent molecular weights of the DEBS proteins were
also evaluated by gel filtration on a Superose 6 HR 10/30
column (Pharmacia), using thyroglobulin (669 kDa) and
apoferritin (443 kDa) as high molecular weight markers.
(see Figure 3A).

25 Recombinant DEBS proteins isolated from cell
extracts were partially purified as described above.
Size exclusion chromatography of a crude extract
containing the three DEBS subunits on Biogel A and
Superose 6 (upper size exclusion limit 15 MDa and 1.5

30 MDa, respectively) revealed that DEBS 1 and 2 associate
more tightly with each other than with DEBS 3. Moreover,
DEBS 1 and 2 (370 kDa and 380 kDa, respectively) elute
over a wide range of fractions corresponding to M_r
between 10 and 1 MDa, indicating that they might form a

35 multimeric complex, which partially dissociates during

gel filtration. DEBS 3, however, is not present in this extremely large M_r range. From size calibration experiments on a Superose 6 column, DEBS 3 (330 kDa) mostly elutes as a dimer (similar to thyroglobulin, 669

5 kDa). Upon concentration, the Biogel A column fractions (see Figure 3A) containing DEBS 1 and 2 alone were found be inactive *in vitro*. However, when pooled with a concentrated fraction containing DEBS 3 alone (Figure 3A), the reconstituted complex of the three proteins

10 showed comparable activity to the DEBS 1, 2, and 3 preparation derived via ammonium sulfate precipitation of the crude cell extract. These purification results suggest that activity of DEBS requires the formation of a high molecular weight oligomeric complex, possibly a

15 trimer of dimers. The formation of homodimers by purified (but not fully active) DEBS subunits has been reported (Aparicio *et al.* *J. Biol. Chem.* (1994), *supra*; Bevitt *et al.* *Eur. J. Biochem.* (1992), *supra*; Caffrey *et al.* *Eur. J. Biochem.* (1991), *supra*; and Leadlay *et al.*

20 *Biochem. Soc. Trans.* (1993), *supra*).

The covalent modification of the cell-free DEBS preparations by ^{14}C-labeled starter units in the absence of chain extension reactions is depicted in Figure 3B. Partially purified DEBS preparations (40 mg total

25 protein) were incubated with the substrates [1-^{14}C]propionyl-CoA (20 μM), [1-^{14}C]butyryl-CoA (160 μM), [1-^{14}C]acetyl-CoA (40 μM), or [1-^{14}C]propionyl-CoA (20 μM) including a 30-min preincubation with iodoacetamide (1 mM). After, denaturation and separation

30 of the proteins in a SDS-PAGE (5%), the separated proteins were electrotransferred onto a nitrocellulose membrane, ^{14}C-labeled proteins were exposed to an X-ray film for 5 days.

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Example 7

Cell-Free Synthesis of 6-dEB and

(2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-

n-heptanoic acid δ-lactone

5   In order to establish an *in vitro* assay system
for polyketide synthesis, partially purified preparations
of the complete DEBS 1, 2, and 3 system and of DEBS
1+2+TE, prepared as described in Example 6, were
incubated with their natural substrates
10   [1-^{14}C]propionyl-CoA, (2RS)-methylmalonyl-CoA, and NADPH.
          The DEBS 1+2+TE and DEBS 1, 2, and 3
preparations described in the Example 6 (purification
Step 2) were adjusted to a concentration of 8 mg total
protein/mL buffer. Incubations were carried out at 28°C
15   with the [1-^{14}C]propionyl-CoA (specific activity 50
Ci/mol, 10 μM), methylmalonyl-CoA (250 μM), and NADPH
(500 μM) dissolved in buffer I in a volume of 250 μL for
3 h. Thereafter, the incubation mix was extracted with 2
x 2 mL ethyl acetate, the ethyl acetate was evaporated
20   of, and the product was analyzed by thin layer
chromatography ("TLC") in 60% ethyl acetate/40% hexane
followed. The TLC plate was exposed to an X-ray film for
2 days.
          Lanes Ia and Ib of the autoradiogram (Figure 4)
25   show extracts of DEBS 1+2+TE including
[1-^{14}C]propionyl-CoA and NADPH but excluding
methylmalonyl-CoA (Ia) and including all 3 substrates
(Ib). Lanes IIa, IIb and IIc of the autoradiogram
(Figure 4) show· incubations of DEBS 1, 2, and 3 including
30   [1-^{14}C]propionyl-CoA and NADPH but excluding
methylmalonyl-CoA (IIa), including all 3 substrates (IIb)
and preincubation of DEBS 1, 2, and 3 with cerulenin (100
μM) for 15 min, followed by addition of all 3 substrates
(IIc). Ethyl acetate/hexane (50:50) was used as the
35   solvent system. In lane Ib the major labeled component

is identical in R_f (0.30) to authentic triketide (2) (see

arrow), while in lane IIb, the least polar labeled

product is identical in R_f (0.40) to authentic 6-dEB (1)

(see arrow). In lanes Ib and IIb the concentrations of

5   the minor products (but not 6-dEB nor the triketide

lactone) vary substantially as a function of the DTT

concentration in the reaction buffer; the structures of

these DTT-dependent products are under investigation.

Control experiments were also performed with DEBS 1+2+TE,

10   with complete DEBS in the absence of NADPH, and with

comparable cell-free preparations from CH999 and from

CH999/pSEK38 (a recombinant strain that expresses the

actinorhodin PKS gene cluster). In all four controls,

neither 6-dEB nor the triketide lactone were detected.

15   The intense, more polar band, evident lanes IIa, IIb and

IIc was also present in all the above null controls

including extracts obtained from CH999 alone. The

identities of the enzymatically generated $[^{14}C]$-(1) and

$[^{14}C]$-(2) were each confirmed by dilution of the

20   respective TLC-purified product with authentic unlabeled

carrier and recrystallization to constant activity. Thus

labeled 6-dEB, from incubation of $[1-^{14}C]$propionyl-CoA

with DEBS 1, 2, and 3, mixed with 15.4 mg of (1), was

recrystallized 4 times from ether/hexane. After each

25   recrystallization, two to three portions of each sample

were analyzed by liquid scintillation counting: 2132 ±

16 dpm/mg (1st recryst); 2117 ± 23 dpm/mg (2nd recryst);

2125 ± 2 dpm/mg (3rd recryst); 2141 ± 17 dpm/mg (4th

recryst); (mean ^{14}C act. 2129 ± 9 dpm/mg). Similarly

30   labeled triketide, from incubation of

$[1-^{14}C]$propionyl-CoA with DEBS 1+2+TE, was mixed with

20.4 mg of unlabeled (2) and recrystallized 4 times from

ether/hexane: 4528 ± 306 dpm/mg (1st recryst); 4725 ± 80

dpm/mg (2nd recryst); 4662 ± 74 dpm/mg (3rd recryst);

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4706 ± 60 dpm/mg (4th recryst); (mean ^{14}C act. 4655±77
dpm/mg).

These results indicate that each cell-free DEBS
protein preparation synthesized a ^{14}C-labeled product
5   with TLC R_f values identical to those of reference
samples of either 6-dEB (**1**) or (2R,3S,4S,5R)-
2,4-dimethyl-3,5-dihydroxy-n-heptanoic acid δ-lactone
(**2**), respectively, as evidenced by TLC-autoradiography
(Figure 4). The identities of [^{14}C]-(**1**) and [^{14}C]-(**2**)
10   were confirmed by dilution of each of the TLC-purified
radiolabeled products with authentic unlabeled carrier
6-dEB or triketide lactone and repeated recrystallization
of each sample to constant activity. The formation of
each lactone product showed an absolute requirement for
15   the relevant protein preparation as well as for
methylmalonyl-CoA and NADPH and was inhibited by both
N-ethylmaleimide and cerulenin, both well-known
inhibitors of the condensation reactions of fatty acid
biosynthesis (Plate, C.A. *et al. J. Biol. Chem.* (1970)
20   245:2868; D'Agnolo, G. *et al. Biochim. Biophys. Acta*
(1973) 326:155; Kauppinen, S. *et al. Carlsberg Res.*
Commun. (1988) 53:357-370). Based on the observed
radiochemical yield of purified product, the formation of
6-dEB catalyzed by DEBS 1, 2, and 3 was estimated to be
25   33 pmol/mg total protein. By comparison, the formation
of (**2**) by DEBS 1+2+TE was 600 pmol/mg total protein.

The specificity of labeling in the triketide
lactone product was unambiguously confirmed by
preparative scale incubation of [1-^{13}C]propionyl-CoA with
30   DEBS 1+2+TE in the presence of methylmalonyl-CoA and
NADPH. Analysis of the derived product (**2a**) (*see* Figure
4 (a)) by 100 MHz ^{13}C NMR showed an enhanced peak at 81.3
ppm corresponding to enrichment at the predicted site,
C-5, in (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-
35

n-heptanoic acid δ-lactone (Kao, C.M. *et al.* *J. Am. Chem. Soc.* (1994), *supra*).

Example 8
Substrate Specificity of DEBS 1+2+TE
In a Cell-Free System

The *in vitro* assays were carried out as described in Example 6, substituting $[1-^{14}C]$propionyl-CoA by either $[1-^{14}C]$butyryl-CoA (160 μM) or $[1-^{14}C]$acetyl-CoA, 40 μM. Incubations of DEBS 1+2+TE (purification Step 2) were performed excluding methylmalonyl-CoA or including methylmalonyl-CoA and NADPH in addition to the appropriate ^{14}C-labeled primer substrate. Alternatively, DEBS 1+2+TE was preincubated with 1 mM N-ethylmaleimide before addition of all 3 substrates. Control experiments carried out in the absence of NADPH as well as with an equivalent protein preparation from *S. coelicolor* CH999 did not yield the observed labeled products. Ethyl acetate/hexane (60:40) was used as the solvent system.

Development of the above radiochromatographic assay has allowed a preliminary analysis of the substrate specificity of these multifunctional enzyme complexes. For example, DEBS 1+2+TE appears to exhibit a relaxed specificity for primer unit analogs, as shown by both protein acylation and product formation. In addition to the expected acylation by $[1-^{14}C]$propionyl-CoA, both DEBS 1+2+TE and DEBS 1 (from the mixture of DEBS 1, 2, and 3) also form covalent adducts with $[1-^{14}C]$acetyl-CoA and $[1-^{14}C]$butyryl-CoA (*see* Figure 3B). Since the protein labeling is unaffected by the presence of active site thiol inhibitors such as iodoacetamide, the substrates are presumably bound to the "loading" acyltransferase domain at the N-terminal end of DEBS 1. All three acyl-CoA substrates appear to react with DEBS 1 or DEBS

1+2+TE with comparable efficiencies. (The apparently
lower intensity of the butyryl-CoA-labeled band in Figure
3B is due to the 10-fold lower specific activity of
$[1-^{14}C]$butyryl-CoA.)   In the presence of
methylmalonyl-CoA and NADPH, both acetyl-CoA and
butyryl-CoA serve as surrogate polyketide chain
initiators for DEBS 1+2+TE, giving rise to compound (4)
(see Figure 6B) the previously described C_8 analog of
compound (2), and what is presumed to be compound (5)
(see Figure 6B), the C_{10} homolog of (2), respectively, as
judged by thin layer chromatography-autoradiography (see
Figure 6A).

In addition to the above CoA thioesters, DEBS
1+2+TE enzyme can also process the N-acetylcysteamine
thioester of the polyketide chain elongation
intermediate, (2S,3R)-2-methyl-3-hydroxypentanoyl-NAC
thioester (3).   Thus incubation of $[1-^{14}C]$-(3) (Cane,
D.E. et al. J. Am. Chem. Soc. (1981) 103:5960; Cane, D.E.
et al. Tetrahedron (1983) 39:3449; Cane, D.E. et al. J.
Am. Chem. Soc. (1986) 108:4957; Cane, D.E. et al. J. Am.
Chem. Soc. (1987) 109:1255; Cane, D.E. et al, Tetrahedron
Lett. (1991) 32:5457; and Cane, D.E. et al. J. Antibiot.
(1995) 48:647-651 (1995)) with DEBS 1+2+TE in the
presence of methylmalonyl-CoA and NADPH gave rise to a
labeled product which had the chromatographic mobility
expected for the triketide lactone (2) and which could be
recrystallized to constant activity when diluted with
unlabeled triketide lactone, as described above.   The
specificity of labeling was unambiguously confirmed by
preparative scale incubation of $[2,3-^{13}C_2]$-(3) with DEBS
1+2+TE, methylmalonyl-CoA and NADPH.   The ^{13}C NMR
spectrum of the resulting triketide lactone (2b) (see
Figure 4) displayed a pair of enhanced and coupled
doublets (J_{CC} = 34.5 Hz) centered at 36.7 and 81.3 ppm,
corresponding to enrichment at each of the expected sites

of labeling, C-4 and C-5, respectively. This result is
consistent with the previously reported incorporation of
$[2,3-^{13}C_2]-(3)$ into the erythromycin macrolide (Cane et
al. J. Am. Chem. Soc. (1981), *supra*; Cane *et al.*

5   *Tetrahedron* (1983), *supra*; Cane *et al. J. Am. Chem. Soc.*
(1986), *supra*; Cane *et al. J. Am. Chem. Soc.* (1987),
supra; Cane *et al. Tetrahedron Lett.* (1991), *supra*) in
intact cell experiments, as well as the results of
numerous experiments in which NAC thioesters of advanced

10   intermediates of polyketide chain elongation have been
shown to be incorporated into other polyketides.

The Examples provided herein lend further
credence to the speculation that these NAC thioesters are
directly loaded on to the appropriate active site in the

15   PKS, and do not require the participation of additional
proteins or cofactors. This experiment also confirms
directly the ability of the macrolide synthase to
recognize exogenously added chain elongation
intermediates and load them correctly on the cognate PKS

20   module for further processing to the natural product.

Example 9
Cell-Free Synthesis of
2,4-Dimethyl-5-ethyl-3-hydroxy-2-pyrone

25   The DEBS1+2+TE preparation described in Example
6 (purification step 2) was used as described in Example
7 but without the addition of NADPH to the reaction
mixture. This reaction produced
2,4-dimethyl-5-ethyl-3-hydroxy-2-pyrone as demonstrated

30   by NMR analysis.

The following Examples provide methods for
inhibiting the synthesis of polyketides in a modular PKS
from natural first-module starter units or from such

35   starter units derived from the decarboxylation of

extender units in so far as such substrates compete with
unnatural starter units. The method involves inhibiting
the loading of the first module of a PKS with a natural
starter unit by inactivating a key active site on which

5   starter units are loaded, for example, by deleting the
KS1 or otherwise rendering KS1 nonfunctional or,
alternatively, by deleting or rendering nonfunctional the
ACP1. In a cell-free system, wherein the synthesis of a
polyketide from unnatural starter units is desired, this

10   method provides the advantage of minimizing undesirable
competitive polyketide synthesis based on the presence of
the natural starter and/or extender units. In addition,
the method spares the extender units which would
otherwise be supplied in a cell-free system at a

15   considerable cost. This method is also of particular
importance in an *in vivo* system in which the production
of desired polyketides from unnatural substrates may be
inhibited by the presence of natural substrates, thereby
precluding the efficient use of the unnatural starter

20   units to yield the desired product.

Example 10
Construction, Expression and Analysis of
[KS1*]-DEBS1+2+TE

25   In the absence of added propionyl-CoA,
DEBS1+2+TE can form the propionyl starter unit through
decarboxylation of methylmalonyl-loaded enzyme. This
reaction requires a functional module 1 KS activity,
which decarboxylates loaded methylmalonate in order to

30   condense the extender unit with the starter unit. In the
absence of supplied propionyl-CoA starter unit, the
decarboxylated extender unit can be transferred
backwards, allowing the loading of a second
methylmalonyl-CoA extender and subsequent formation of a

35   diketide. As this back-formation of propionyl units is

undesirable when alternative starter units are being
supply to the system, a mutant of DEBS1+2+TE in which
the module 1 KS has been inactivated through
site-directed mutagenesis was prepared. The KS1 sequence

5   was altered such that the active site cysteine residue
(in the signature sequence cys-ser-ser-ser-leu) was
replaced by alanine. The resulting expression plasmid,
designated pKAO179, encodes a 2-module PKS which is
inactive under the standard reaction conditions

10   (propionyl-CoA, methylmalonyl-CoA, and NADPH).
Inactivation of KS1 thus prevents the back formation of
propionyl units, but also prevents diketide formation.
When this protein is supplied with diketide thioester,
i.e., (2S,3R)-2-methyl-3-hydroxy-pentanoyl

15   N-acetylcysteamine thioester, methylmalonyl-CoA, and
NADPH, however, the triketide product
(2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxyheptanoic
δ-lactone is produced. This construct allows the *in
vitro* production of triketides having unusual starter

20   units through use of the corresponding diketide
thioesters, uncontaminated by the normal triketide
product.

Example 11
In Vivo Production of Novel Polyketides
25   by Fermentation Using [KS1*]DEBS Mutants
As described in Example 10, the module 1 β-
ketoacylsynthase (KS1) activity of DEBS can be
inactivated through site-directed mutagenesis. This

30   mutation can be introduced into any combination of
modules to produce a set of DEBS-backed PKSs which are
incompetent for polyketide synthesis unless supplied with
a suitable diketide thioester, e.g., 2-methyl-3-
hydroxypentanoyl N-acetylcysteamine thioester or analogs.

35   The method described in Example 10 can be extended to

allow for the *in vivo* production of novel polyketides through feeding of the appropriate diketide thioester analogs to actively growing cultures of *S. coelicolor* CH999 containing [KS1*]-DEBS-based expression plasmids.

5   The corresponding diketide as a free carboxylic acid may also be fed to the cultures if the cellular thioesterification system is functional on the acid, and if the cells are permeable to the acid. For example, the *in vivo* production of (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-

10   dihydroxy-n-heptanoic acid δ-lactone is described above.

A culture of *S. coelicolor* CH999/pKAO179 is established by inoculation of 200 mL of SMM medium (5% PEG-800, 0.06% $MgSO_4$, 0.2% $(NH_4)_2SO_4$, 25 mM TES, pH 7.2, 25 mM KH_2PO_4, 25 mM K_2HPO_4, 1.6% glucose, 0.5% casamino

15   acids, trace elements) with spores. The culture is incubated at 30°C with shaking at 325 rpm. A solution of (2S,3R)-2-methyl-3-hydroxypentanoyl N-acetlycysteamine thioester (100 mg) and 4-pentynoic (15 mg) in 1 mL of methylsulfoxide is added to the culture in three parts:

20   after 50 hours (400 mL); after 62 hours (300 mL); and after 86 hours (300 mL). After a total of 144 hours, the culture is centrifuged to remove mycelia. The fermentation broth is saturated with NaCl and extracted with ethyl acetate (5 x 100 mL). The combined organic

25   extract is dried over Na_2SO_4, filtered, and concentrated. Silica gel chromatography yields (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-n-heptanoic acid δ-lactone.

This method provides a means for large-scale production of novel modular polyketides containing

30   unnatural starter units, uncontaminated by the polyketide containing the native propionate starter unit.

The cell-free results reported here, in conjunction with the availability of facile mutagenesis

35   tools, provide a novel approach to the study of modular

PKS structure and mechanisms. The considerable yield of fully active protein from the recombinant source described will permit detailed analysis of this multifunctional catalyst by radioisotopic methods as well

5 as by NMR and mass spectroscopy. Given that DEBS can accept a variety of substrates as primers, it will be possible to make quantitative assessments of substrate specificity by determination of the relevant steady state kinetic parameters and to further probe mechanistic

10 details. In addition, cell-free systems such as the one reported here provide a completely novel route for the controlled synthesis of novel polyketides which might otherwise not be accessible via *in vivo* engineered biosynthesis.

15 Thus, novel methods for producing polyketides, are disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention

20 as defined by the appended claims.

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